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(57) Abstract

The present invention relates to the field of hematopoiesis in mammals, and more particularly to the use of certain novel compounds and known compounds for the *in vitro*, *ex vivo*, or *in vivo* expansion of hematopoietic cells.

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COMPOSITIONS AND METHODS FOR EXPANSION OF HEMATOPOIETIC CELLS

FIELD OF THE INVENTION

The present invention relates to the field of hematopoiesis in mammals, and more particularly to the use of certain novel compounds and known compounds for the *in vitro, ex vivo,* or *in vivo* expansion of hematopoietic cells.

BACKGROUND OF THE INVENTION

Certain chemotherapeutic and radiation treatments for cancer and other serious illness have the undesired side effect of killing all dividing and differentiating cells. This effect is most severe on the differentiating cells of the hematopoietic system, which are essential for repopulating the bone marrow and permitting the redevelopment of a functioning immune system following such treatment. Normally, bone marrow toxicity or myelosuppression is the limiting factor in the use of such treatments. The acute effects of myelosuppression are the loss of neutrophils (neutropenia) and the loss of platelets (thrombocytopenia) in the blood. These conditions increase patient susceptibility to infections and hemorrhagic complications.

Several strategies have been used to try to alleviate this problem. Stem cell transplantation after cytotoxic therapy has been used to replace hematopoietic stem cells that are killed during treatment. The goal of this therapy is to allow these cells to self renew and also differentiate to mature blood cells. Recently, bone marrow has been replaced by peripheral blood as a source of stem cells for transplantation. Since the frequency of stem cells is quite low in peripheral blood, recombinant growth factors are used as conditioning regiments to mobilize cells from the bone marrow and increase their frequency in the blood. However, even after mobilization several apheresis are needed to collect sufficient amounts of stem cells.

One way to increase stem cell numbers is to expand them *ex vivo* with various growth factors. While expansion of progenitor cells at different stages has been reported, (Haylock DN, To LB, Dowse TL, Juttner CA, Simmons PJ, Blood 80:1405, 1992; Brugger W, Mocklin W, Heimfeld S Berenson RJ , Mertelsmann R, Kanz L, Blood 81:2579, 1993; Srour EG, Brandt JE, Briddell RA, Grigsby S, Leemhuis T, Hoffman R Blood 81:661, 1993) these cells will eventually completely differentiate and die in culture. The use of an agent that will allow expansion up to specific point of differentiation could allow an

accumulation of progenitor cells without further maturation. So far, compounds such as MIP-1a and TGF-β have been used in expansion cultures with limited success (Verfaillie CM, Catanzarro PM, Li WN, J-Exp-Med., 179(2): 643-9. 1994; Mayani H, Little M-T, Dragowska W, Thornbury G, Lansdorp PM, Exp Hematol 23:422 1995) While it may be necessary to transplant stem cells for long term engraftment, more mature progenitors may be important for short term recovery. If committed progenitors such as CFU-GM could be expanded it might be possible to use these cells to shorten the time for neutrophil recovery and possibly reduce the risks of infection post chemotherapy.

SUMMARY OF THE INVENTION

In one aspect, the invention provides a method for expanding the population of a culture of myelopoietic progenitor cells *in vitro* by (1) adding to the cell culture medium containing hematopoietic growth factors an effective amount of a compound of formula (I) to reversibly reduce the rate of differentiation of the cells, then (2) removing the compound of Formula (I) after a period of 1-100 days, preferably 1-7 days; and (3) transplanting the cells into a patient in need thereof.

In another aspect, the invention provides a method for expanding the population of myelopoietic progenitor cells *in vivo* by administering to a patient in need thereof, an effective amount of a compound of Formula (I).

A further aspect of the invention provides a method for reversibly inhibiting myelopoiesis in mammalian tissue *in vivo* and *ex vivo* comprising administering to said tissue an amount of a compound of formula (I)

Additional aspects of this invention include assay methods for use in identifying or screening for compounds which reversibly inhibit hematopoietic progenitor cells, as described in detail below.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof

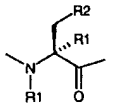
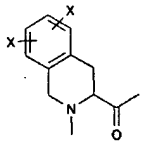
DETAILED DESCRIPTION OF THE INVENTION

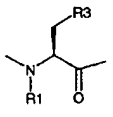
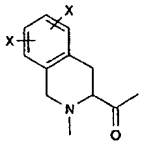
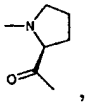
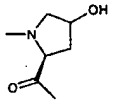
The present invention provides a method for expanding the population of myelipoietic progenitor cells *in vitro* comprising adding to a culture medium with hematopoietic growth factors an amount of a compound which is glycinamide (H-Gly-NH_2) or any tetra-, tri- or dipeptide analog which can be converted in serum to glycinamide (H-Gly-NH_2). Preferred compounds are those of Formula (I)

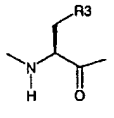
H-A-B-C-Gly-NH_2

(I)

wherein:

A is a bond,  , or  ;

B is a bond,  ,  ,  ,  , or  ;

C is a bond or  ;

R1 is H or C_{1-4}alkyl;

R2 is H,  , naphthyl, indoyl, or pyridyl;

R3 is H, C_{1-4}alkyl, cycloC_{3-8}alkyl,  , naphthyl, indoyl, or pyridyl; wherein the C_{1-4} alkyl may be substituted by one or more X groups;

X is H, OH, I, Br, NH_2, N_3, O-C_{1-4}alkyl, C_{1-4}alkyl, or COPh;

or a pharmaceutically acceptable salt thereof.

Specific examples of such peptides include the following:

H-Gly-NH_2

H-Tyr-Pro-Leu-Gly-NH_2 [SEQ ID NO: 1]

H-Tic-Leu-Gly-NH_2

H-Tyr-Pro-Trp-Gly-NH_2 [SEQ ID NO: 2]

H-Tyr-Pro-Phe-Gly-NH_2 [SEQ ID NO: 3]

H-Phe-Pro-Leu-Gly-NH_2 [SEQ ID NO: 4]

Hpp-Pro-Leu-Gly-NH_2 [SEQ ID NO: 5]

H-(3-I)Tyr-Pro-Leu-Gly-NH_2 [SEQ ID NO: 6]

H-(4-NH_2)Phe-Pro-Leu-Gly-NH_2 [SEQ ID NO: 7]

H-Tyr-$^{\Delta 3,4}$Pro-Leu-Gly-NH_2 [SEQ ID NO: 8]

H-(4-N_3)Phe-Pro-Leu-Gly-NH_2 [SEQ ID NO: 9]

H-Tyr-Ala-Leu-Gly-NH_2 [SEQ ID NO: 10]

H-Tic-Pro-Leu-Gly-NH_2 [SEQ ID NO: 11]

H-(4-Br)Phe-Pro-Leu-Gly-NH_2 [SEQ ID NO: 12]

H-Nal-Pro-Leu-Gly-NH_2 [SEQ ID NO: 13]

H-Trp-Pro-Leu-Gly-NH_2 [SEQ ID NO: 14]

H-Tyr-NMeAla-Leu-Gly-NH_2 [SEQ ID NO: 15]

H-(3,5-I)Tyr-Pro-Leu-Gly-NH_2 [SEQ ID NO: 16]

H-Pal-Pro-Leu-Gly-NH_2 [SEQ ID NO: 17]

H-Tyr-Leu-Gly-NH_2

H-Tyr-Hyp-Leu-Gly-NH_2 [SEQ ID NO: 18]

H-MeTyr-Pro-Leu-Gly-NH_2 [SEQ ID NO: 19]

H-Tic(7-OH)-Leu-Gly-NH_2 [SEQ ID NO: 20]

H-Bpa-Pro-Leu-Gly-NH_2 [SEQ ID NO: 21]

H-Tyr-Pro-Lys-Gly-NH_2 [SEQ ID NO: 22]

H-Tyr-hPro-Leu-Gly-NH_2 [SEQ ID NO: 23]

H-Phe-Leu-Gly- NH_2

H-Trp-Leu-Gly- NH_2

H-Leu-Gly- NH_2

Most preferred is glycinamide (H-Gly-NH_2).

Also included in this invention are pharmaceutically acceptable salt complexes of the compounds of this invention.

All alkyl groups may be straight or branched. The compounds of the present invention may contain one or more asymmetric carbon atoms and may exist in racemic and optically active form. All of these compounds and diastereoisomers are contemplated to be within the scope of the present invention.

The peptides of the invention are prepared by the solid phase technique of Merrifield, J. Am. Chem. Soc., 85:2149 (1964), or solution methods known to the art may be successfully employed. The methods of peptide synthesis generally set forth in J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis", Pierce Chemical Company, Rockford, IL (1984) or M. Bodansky, Y. A Klauser and M. A. Ondetti, "Peptide Synthesis", John Wiley & Sons, Inc., New York, NY (1976) may be used to produce the peptides of this invention and are incorporated herein by reference.

Each amino acid or peptide is suitably protected as known in the peptide art. For example, the α-fluoroenylmethyloxycarbonyl group (Fmoc) or t-butoxycarbonyl (t-Boc) group are preferred for protection of the amino group, especially at the α-position. A suitably substituted carbobenzoxy group may be used for the ε-amino group of lysine and benzyl group for the β and γ carboxy groups of Asp and Glu respectively. Suitable substitution of the carbobenzoxy protecting group is ortho and/or para substitution with chloro, bromo, nitro or methyl, and is used to modify the reactivity of the protective group. Except for the t-Boc group, the protective groups are, most conveniently, those which are not removed by mild acid treatment. These protective groups are removed by such methods as catalytic hydrogenation, sodium in liquid ammonia or HF treatment as known in the art.

If solid phase synthetic methods are used, the peptide is built up sequentially starting from the carboxy terminus and working toward the amino terminus of the peptide. Solid phase synthesis is begun by covalently attaching the C terminus of a protected amino acid to a suitable resin, such as benzhydrylamine resin (BHA), methylbenzhydrylamine resin (MBHA) or chloromethyl resin (CMR), as is generally set forth in U. S. Patent No. 4,244,946 or phenyl acid amino methyl resin (PAM). A BHA or MBHA support resin is used if the carboxy terminus of the product peptide is to be a carboxamide. A CMR or PAM resin is generally used if the carboxy terminus of the product peptide is to be a carboxy group, although this may also be used to produce a carboxamide or ester.

The protective group on the α-amino group is removed by mild acid treatment (i.e. trifluoroacetic acid). Suitable deprotection, neutralization and coupling cycles known in the art are used to sequentially add amino acids without isolation of the intermediate, until the desired peptide has been formed. The completed peptide may then be deblocked and/or split from the carrying resin in any order.

Treatment of a resin supported peptide with HF or HBr/acetic acid splits the peptide from the resin and produces the carboxy terminal amino acid as a carboxylic acid or carboxamide.

If an ester is desired, the CMR or PAM resin may be treated with an appropriate alcohol, such as methyl, ethyl, propyl, butyl or benzyl alcohol, in the presence of triethylamine to cleave the peptide from the resin and product the ester directly. Esters of the peptides of this invention may also be prepared by conventional methods from the carboxylic acid precursor. Typically, the carboxylic acid is treated with an alcohol in the presence of an acid catalyst. Alternatively, the carboxylic acid may be converted to an activated acyl intermediate, such as an acid halide, and treated with an alcohol, preferably in the presence of a base.

The preferred method for cleaving a peptide from the support resin is to treat the resin supported peptide with anhydrous HF in the presence of a suitable cation scavenger, such as anisole or dimethoxybenzene.

This method simultaneously removes all protecting groups, except a thioalkyl group protecting sulfur, and splits the peptide from the resin. Peptides hydrolyzed in this way from the CMR and PAM resins are carboxylic acids, those split from the BHA resin are obtained as carboxamides.

Modification of the terminal amino group of the peptide is accomplished by alkylation or acylation by methods generally known in the art. These modifications may be carried out upon the amino acid prior to incorporation into the peptide, or upon the peptide after it has been synthesized and the terminal amino group liberated, but before the protecting groups have been removed.

Typically, acylation is carried out upon the free amino group using the acyl halide, anhydride or activated ester, of the corresponding alkyl or aryl acid, in the presence of a tertiary amine. Mono-alkylation is carried out most conveniently by reductive alkylation of the amino group with an appropriate aliphatic aldehyde or ketone in the presence of a mild reducing agent, such a lithium or sodium cyanoborohydride. Dialkylation may be carried out by treating the amino group with an excess of an alkyl halide in the presence of a base.

Solution synthesis of peptides is accomplished using conventional methods used to form amide bonds. Typically, a protected t-Boc amino acid which has a free carboxyl group is coupled to a protected amino acid which has a free amino group using a suitable coupling agent, such as N,N'-dicyclohexyl carbodiimide (DCC), optionally in the presence of catalysts such as 1-hydroxybenzo-triazole (HOBT) or dimethylamino pyridine (DMAP).

Other methods, such as the formation of activated esters, anhydrides or acid halides, of the free carboxyl of a protected t-Boc-amino- acid, and subsequent reaction with the free amine of a protected amino acid, optionally in the presence of a base, are also suitable. For example, a protected Boc-amino acid or peptide is treated in an anhydrous solvent, such as methylene chloride or tetrahydrofuran (THF), in the presence of a base, such as N-methyl morpholine, DMAP (dimethylaminopyridine) or a trialkyl amine, with isobutyl chloroformate to form the "activated anhydride", which is subsequently reacted with the free amine of another protected amino acid or peptide. The peptide formed by these methods may be deprotected selectively, using conventional techniques, at the amino or carboxy terminus and coupled to other peptides or amino acids using similar techniques. After the peptide has been completed, the protecting groups may be removed as hereinbefore described, such as by hydrogenation in the presence of a palladium or platinum catalyst, treatment with sodium in liquid ammonia, hydrofluoric acid or alkali.

If the final peptide, after it has been deprotected, contains a basic group, an acid addition salt may be prepared. Acid addition salts of the peptides are prepared in a standard manner in a suitable solvent from the parent compound and a slight excess of an acid, such a hydrochloric, hydrobromic, sulfuric, phosphoric, acetic, maleic, succinic or methanesulfonic. The acetate salt of the peptide is generally useful. If the final peptide contains an acidic group, cationic salts may be prepared. Typically the parent compound is treated with a slight excess of an alkaline reagent, such as a hydroxide, carbonate or alkoxide, containing the appropriate cation. Cations such as Na^+, K^+, Ca^{++} and NH4+ are examples of cations present in pharmaceutically acceptable salts. Na^+ and NH4+ may be preferred.

A compound of this invention is added to a culture medium containing one or more hematopoietic growth factor in an amount effective to reversibly reduce the rate of differentiation of the cells. The amount of peptide will greatly depend on the composition and cell count of the culture. As one example, a suitable amount of peptide is between about 0.1 and about 500 micrograms/mL culture; preferably 10-100 micrograms/mL *ex vivo*, or 0.1-100 milligrams/kg *in vivo*.

Examples of hematopoietic growth factors are M-CSF, IL-1, IL-3, IL-6, IL-11, G-CSF, GM-CSF, LIF, TGF-β, MIP1-α, FLT3 ligand, SCF, TPO, MPIF-1, IL-3/GM, IL-3/G, FLT3/GM. Preferred growth factors are: combinations of SCF, IL-11; IL-3, IL-6; IL-1, G-CSF, GM-CSF, TPO, FLT3, M-CSF

The compounds of Formula (I) have a characteristic biological activity of reversibly inhibiting myelopoiesis in mammals, mammalian tissue, or other biological samples *in vitro*, *ex vivo* or *in vivo*. These compounds may almost completely inhibit differentiation of myelopoietic cells, e.g., colony forming units in culture (CFU-C), such as CFU-GM, when in contact with such cells.

The compounds of this invention are useful in providing a protective function in cancer or other therapies which involve irradiation and/or cytotoxic drugs which function normally to kill proliferating cells, including proliferating stem cells. The inventors have discovered that certain compounds are capable of inhibiting stem cell differentiation when the compounds are in contact with the stem cells (thereby causing undifferentiated cells to pile up). These compounds also have a desirable attribute of reversing the inhibition by simply removing the compound from contact with the stem cells. All of the compounds of this invention have a reversible action, i.e., they protect the stem cells from being killed when the cells are in contact with the peptides during insult and, following the insult, when the compound is no longer administered, the inhibitory effect ceases, thus allowing greater numbers of stem cells to be present and capable of normal proliferation and differentiation. The myeloprotectant compounds of this invention are also specific to normal hematopoietic stem cells and inhibit the proliferation of normal cells and not the proliferation of target cancer cells.

This invention provides a method for reversibly inhibiting myelopoiesis in mammals, mammalian tissue, including bone marrow and other samples, *in vivo* and *ex vivo* and *in vitro*. This method entails administering to the mammal, tissue or sample an amount of a peptide of the formula (I) or glycinamide, effective to reversibly inhibit the formation of myelopoietic colonies during the time the mammal, tissue or bone marrow is exposed to myelosuppressive therapy. In this method, the myelopoietic colonies inhibited are CFU-C colony forming cells, and more specifically, CFU-GM colony forming cells.

This method may be accomplished *in vivo*. In other words, a mammalian subject, preferably a human patient, undergoing chemotherapy or radiation may be administered the peptide so that the reversible inhibition of myelopoiesis occurs *in vivo*.

Alternatively, the method may be accomplished *ex vivo* or *in vitro*. According to this embodiment of the method, the mammalian tissue, for example, bone marrow, is contacted *ex vivo* with an effective amount of myeloprotectant peptide of this invention substantially simultaneously with exposure of the tissue to radiation or chemotherapeutics.

Inhibition of myelopoiesis is reversible when administration of the insult and the peptide, which occur *ex vivo*, cease.

Thus, any of the myeloprotectant peptides described above in formula (I), when administered prophylacticly and/or therapeutically as a myelosuppressive treatment, benefit the recipient by protecting the normal hematopoietic stem cells from the damaging agent(s). Treatment with a myeloprotectant of this invention may be necessary throughout the time period in which the insulting agent is present within the body. Inhibition of normal hematopoietic stem cells during this period could protect normal cells while damaging only target cells such as cancer cells.

Because the peptides of this invention have demonstrated reversible inhibition, a more rapid rebound of hematopoietic stem cell proliferation is expected when administration of the myeloprotectant peptide is ceased (concurrently with, or shortly after administration of the chemotherapeutic or radiation is ceased). The end result of treatment with the peptides of this invention as myeloprotective agents is to prevent neutropenia and or accelerate neutrophil/leukocyte recovery, and prevent stem cell loss due to myelosuppression. The advantage of such administration to the patient includes fewer infections due to leukopenia and the ability to use more aggressive chemotherapy regimens to kill cancer cells (dose intensification/increase dose frequency).

As used throughout the description of the methods for inhibiting myelopoiesis, the term "effective amount" refers to that amount of the peptide of formula (I) which evokes a reversible inhibition of proliferating myelopoietic cells. In general, in order to exert an inhibitory effect, the peptides of the invention may be administered to human or other mammalian patients by injection in the dose range of about 0.5 mg to about 1000 mg. As one example, a desirable dosage range is from about 5 to about 1000 mg. Oral administration dosages are desirably in the range of about 5 mg to about 2000 mg, desirably, for example, about 10 mg to 1000 mg per 70 kg body weight per day. If administered by infusion or similar techniques, the dose may be in the range of about 5 micrograms to about 200 mg per 70 kg body weight, for example about 100 micrograms to 100 mg over six days. In principle, it is desirable to produce a concentration of the peptide of about 10^{-10}M to about 10^{-5}M in the extracellular fluid of the patient, preferably 10^{-8}M.

For adaptation of the method for *ex vivo* or *in vitro* use of these peptides, e.g., for administration to bone marrow outside of the body of the patient donor, the effective amounts or dosages may be adjusted based on the amount of tissue being treated. Preferably, an effective *in vitro* concentration is in the range of about 10^{-8}M to 10^{-5}M.

One of skill in the art may readily determine other appropriate dosages, depending on the mode of administration, and the level of aggressiveness of therapy required in the specific circumstance.

According to a still further feature of the present invention there are provided pharmaceutical compositions comprising as active ingredient one or more peptides of formula (I) as hereinbefore defined or physiologically compatible salts thereof, in association with a pharmaceutical carrier or excipient. The compositions according to the invention may be presented, for example, in a form suitable for oral, nasal, parenteral or rectal administration.

As used herein, the term "pharmaceutical" includes veterinary applications of the invention. These peptides may be encapsulated, tableted or prepared in an emulsion or syrup for oral administration. Pharmaceutically acceptable solid or liquid carriers may be added to enhance or stabilize the composition, or to facilitate preparation of the composition. Liquid carriers include syrup, peanut oil, olive oil, glycerin, saline and water. Solid carriers include starch, lactose, calcium sulfate dihydrate, terra alba, magnesium stearate or stearic acid, talc, pectin, acacia, agar or gelatin. The carrier may also include a sustained release material such as glyceryl monostearate or glyceryl distearate, alone or with a wax. The amount of solid carrier varies but, preferably will be between about 20 mg to about 1 g per dosage unit.

The pharmaceutical preparations are made following the conventional techniques of pharmacy involving milling, mixing, granulating, and compressing, when necessary, for tablet forms; or milling, mixing and filling for hard gelatin capsule forms. Capsules containing one or several active ingredients may be produced, for example, by mixing the active ingredients with inert carriers, such as lactose or sorbitol, and filling the mixture into gelatin capsules. When a liquid carrier is used, the preparation will be in the form of a syrup, elixir, emulsion or an aqueous or non-aqueous suspension. Such a liquid formulation may be administered directly p.o. or filled into a soft gelatin capsule. Organ specific carrier systems may also be used.

Alternately pharmaceutical compositions of the peptides of this invention, or derivatives thereof, may be formulated as solutions of lyophilized powders for parenteral administration. Powders may be reconstituted by addition of a suitable diluent or other pharmaceutically acceptable carrier prior to use. The liquid formulation is generally a buffered, isotonic, aqueous solution. Examples of suitable diluents are normal isotonic saline solution, standard 5% dextrose in water or buffered sodium or ammonium acetate

solution. Such formulation is especially suitable for parenteral administration, but may also be used for oral administration and contained in a metered dose inhaler or nebulizer for insufflation. It may be desirable to add excipients such as polyvinylpyrrolidone, gelatin, hydroxy cellulose, acacia, polyethylene glycol, mannitol, sodium chloride or sodium citrate.

A pulverized powder of the peptides of this invention may be combined with excipients such as cocoa butter, glycerin, gelatin or polyethylene glycols and molded into a suppository for rectal administration. The pulverized powders may also be compounded with an oily preparation, gel, cream or emulsion, buffered or unbuffered, and administered through a transdermal patch. Nasal sprays may be formulated similarly in aqueous solution and packed into spray containers either with an aerosol propellant or provided with means for manual compression.

Dosage units of such pharmaceutical compositions containing the compounds of this invention preferably contain about 1 microgram to 2 g, for example, 1 microgram to about 50 mg of the peptide of formula (I) or salt thereof.

ASSAYS

The following assays may be used to demonstrate the reversible inhibition of myeleopoiesis and expansion of hematopoietic cells.

Cell preparation : BDF1 female mice were sacrificed and bone marrow was harvested from femurs by flushing with medium(McCOY's supplemented with 15% fetal calf serum) using a 10mL syringe and a 26 gauge needle. In some experiments, cells were incubated at 37°C for 90 min. in polystyrene tissue culture flasks to remove plastic adherent cells. Low density cells were obtained by spinning cells through optiprep™ 1.077g/mL separation medium (Life Technologies Inc.) for 15 min. at 800g and 4°C.

For enrichment of cells with the Sca-1 phenotype, low density cells were positively selected with antibody coupled to magnetic beads using the MACS Sca-1 MultiSort Kit (Miltenyl Biotech) according to the manufacturers instructions.

Expansion cultures : Unseparated or Sca-1 enriched bone marrow cells were incubated in medium (McCOY's medium supplemented with 15% fetal calf serum) polystyrene tissue culture flasks or 24 well plates. Combinations of the following growth factors were added to cultures: murine interleukin-1, interleukin-3, interleukin-6, GM-CSF, stem cell factor, human G-CSF. Growth factors were added to give a final concentration of

10 ng./mL (50 ng./mL for stem cell factor) Glycinamde was added at the same time as growth factors at concentrations ranging from 7.5 to 60 ug./mL. Cultures were incubated for four to thirteen days at 37°C in 5% O_2 and 6% CO_2

Progenitor cell assays: Cells from expansion cultures were washed twice and mixed with 0.3% agar in medium with growth factors. This was added to Falcon 12 well plates at 0.5 mL. per well at cell concentrations ranging from 5 x 10^4 per well to 160 cells per well. Plates were left at room temperature for about 10 min. for agar to gel and were then placed in 37° C incubator at 5% O_2, and 6% CO_2.

CFU-GM responsive to GM-CSF (10 ng/mL) were counted after seven days as colonies greater than fifty cells per colonies.

HPP(high proliferative potential) responsive to interleukin 3 (10 ng/mL), interleukin 1 (10 ng./mL) and stem cell factor (50 ng/mL) were counted after fourteen days as colonies greater than 0.5 mm in diameter with one or more dense centers. All other colonies grown with these factors with at least fifty cells were counted as LPP(low proliferative potential)

CFU inhibition assay: In Falcon 12 well plates, unseparated or non-adherent bone marrow cells (5 X10^4 cells/well) were mixed with medium and 0.3% agar , M-CSF (200 U/mL) and glycinamide (7.5 to 60 ug/mL). After 7 days at 37°C. 6%CO_2 a colony with greater than 50 cells was counted as 1 CFU-GM. CFU inhibition was determined by comparing cultures with glycinamide to cultures with 0.1% BSA/PBS. All sample was assayed in triplicate.

Human lung carcinoma cell line inhibition assay: In 12 well plates , A549 cells (ATCC CCL-185) were added at 125 cells/well in McCOY's medium with 20% fetal bovine serum in 0.3% agar. After 10 ten days at 37°C , 6% CO_2 , colonies of greater than 50 cells were counted.

Micro-Inhibition Assay: One screening and identification method useful in the present invention may employ the step of screening test samples which detectably bind to a receptor for *in vitro* or *in vivo* inhibition of CFU-C colony formation in either a conventional 7 day CFU-GM assay (CFU-C Assay), well known to those skilled in the art, or in the novel, micro-screening assay as described herein. The micro-inhibition assay is a modification of the conventional assay and provides for an efficient and rapid screening and identification of inhibitors. The presence or amount of inhibition of CFU-C or CFU-GM colony formation can then be measured in order to identify those test samples which

act as agonists. For example, in one such embodiment, an assay for the screening or identification of other myeloprotectant compounds capable of reversible inhibition includes the following steps. First, to measure the occurrence of, and degree of, inhibition, a test sample containing one or more test compounds and selected hematopoietic growth factors is contacted with bone marrow cells in soft agar (a semi-solid matrix) in a 96 well tissue culture plate. The hematopoietic growth factor may be any of the known factors. Desirable factors specifically include M-CSF, IL-1, IL-3, IL-6,G-CSF,GM-CSF,FLT3 stem cell factor (SCF), and/or a combination thereof.

The plate is then incubated under conditions which would permit the detection of inhibition of CFU-C CFU-C or CFU-GM colony growth. Such conditions include incubation at about 37°C humidified atmosphere of about 7% CO_2 for about 4 to 14 days. The degree of inhibition caused by the test sample is detected by measuring the conversion of the mitochondrial metabolism of (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), also known as MTT, to an insoluble formazan crystal which can be dissolved in sodium dodecyl sulfate (SDS). Optical density is measured at 570 nm with a reference filter of 750 nm on an ELISA reader. The results of the assay with the compound are compared to those of a control, i.e., bone marrow cells with growth factors and no compound, as well as marrow cells only. Increased OD means increased MTT conversion indicative of increased cell metabolism or growth.

Micro-Reversibility Assay: An alternative assay method, called a pre-CFU-liquid culture (PCLC) assay, enables the determination of whether the test compound demonstrates reversibility of inhibition. In the PCLC assay or the novel micro reversibility assay as described below, the test sample is first contacted with bone marrow cells in a liquid medium with or without a test compound, in this instance a compound of formula (I), in a 96 well tissue culture plate for 1 to 4 days. Marrow cells are washed with medium three times and recultured in either a conventional CFU-C assay or the above noted Micro-Inhibition assay. The degree of inhibition caused by the test sample is again detected by measuring MTT conversion, as an index of CFU-C proliferation, and measuring optical density at 570 nm with a reference filter of 750 nm on an ELISA reader, using the same controls, as described above.

The assay methods may be further modified by preparing two or more additional test samples from the original test sample or samples that are determined to inhibit CFU-C colony formation activity. These additional test samples contain a lesser number of test compounds than the original test sample from which they were prepared. The steps of the

assays may then be repeated as many times as desired or until the test compound or compounds which bind to the receptor preparation have been identified.

The following Examples illustrate the preparation and uses of myeloprotective peptides of this invention. For ease of illustration, a single exemplary compound of formula (I) was employed in the assays. It should be understood, however, that other peptides falling within formula (I) demonstrate similar biological activity. These examples are illustrative only, and do not limit the scope of the present invention.

The abbreviations used are as folows:

Tic = 2-carboxytetrahydroquinoline

HPP = 3-[(4-hydroxy)-phenyl]propionic acid

(3-I)Tyr = 3-iodotyrosine

(4-NH_2)Phe = 4-aminophenylalanine

$\Delta 3,4$Pro = 3,4 -dehydroproline

(4-N_3)Phe = 4-azidophenylalanine

(4-Br)Phe = 4-bromophenylalanine

Nal = 2-naphthylalanine

NMeAla = N-methylalanine

(3,5-I)Tyr = 3,5-diiodotyrosine

Pal = 3-pyridylalanine

Hyp = trans-4-hydroxyproline

MeTyr = alpha-methyltyrosine

Tic(7-OH) = 2-carboxy-7-hydroxytetrahydroisoquinoline

Bpa= benzoylphenylalanine

hPro = pipecolinic acid

Example 1

Preparation of H-Phe-Pro-Leu-Gly-NH_2 [SEQ ID NO:4]

A. **Boc-Phe-Pro-Leu-Gly-NH_2**

A solution of H-Pro-Leu-Gly-NH_2 (100 mg, 0.341 mmol; Bachem Bioscience Inc.) in dimethyl formamide (DMF) was treated with Boc-Phe-OH (100 mg, 0.375 mmol; Advanced Chemtech), 1-hydroxybenzotriazole (HOBt) (51 mg, 0.375 mmol), diisopropylethylamine (DIEA) (119 microliters, 0.682 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide.HCl (EDC•HCl) (72 mg, 0.375 mmol) at room temperature for 24 hours. The reaction mixture was evaporated and the residue purified by flash chromatography (silica gel, 5% MeOH in $CHCl_3$) to give 220 mg of the title compound. MS (ES) m/e 532 $[M+H]^+$; 530 $[M-H]^-$.

B. **H-Phe-Pro-Leu-Gly-NH_2**

The compound of Example 1A (220 mg) was treated with 4N HCl/dioxane at room temperature for 2 hours. The reaction mixture was evaporated and purified by a bond elute column [0.1% aqueous trifluoroacetic acid (TFA)] to give 130 mg of the title compound. MS (FAB) m/e 432 $[M+H]^+$; HPLC k' 2.96 (PRP-1, gradient, A:0.1% aqueous TFA B:CH_3CN-0.1% TFA, 5-50% B over 30 minutes, UV detection at 220 nm).

Example 2

Preparation of H-(3-I)-Tyr-Pro-Leu-Gly-NH_2 [SEQ ID NO:6]

A. **Boc-(3-I)-Tyr(3BrBzl)-Pro-Leu-Gly-NH_2**

A solution of H-Pro-Leu-Gly-NH_2 (100 mg, 0.341 mmol; Bachem Bioscience Inc.) in DMF was treated with Boc-(3-I)-Tyr(3BrBzl)-OH (215 mg, 0.375 mmol; Peninsula Labs), HOBt (51 mg, 0.375 mmol), DIEA (119 microliters, 0.682 mmol) and EDC•HCl (72 mg, 0.375 mmol) at room temperature for 48 hours. The reaction mixture was evaporated and the residue purified by flash chromatography [(silica gel, 15% MeOH in ethyl acetate (EtOAc)] to give 350 mg of the title compound. MS (ES) m/e 842 $[M+H]^+$; 840 $[M-H]^-$.

B. H-(3-I)-Tyr-Pro-Leu-Gly-NH_2

The compound of Example 2A (350 mg) was treated with TFA at room temperature for 30 minutes. The reaction mixture was evaporated and the residue treated with anhydrous hydrogen fluoride (HF) at 0°C for 1 hour. The residue after evaporation of the HF was precipitated from ether. Purification by preparative high pressure liquid chromatography (HPLC) (PRP-1 packing material [Hamilton], 12% CH_3CN in H_2O with 0.1% TFA) gave 78 mg of the title compound. MS (ES) m/e 573 $[M+H]^+$; HPLC k' 4.55 (PRP-1, gradient, A: 0.1% aqueous TFA, B: CH_3CN-0.1% TFA, 5-50% B over 30 minutes, UV detection at 220 nm).

Example 3

Preparation of H-Tyr-Pro-Phe-Gly-NH_2 [SEQ ID NO:3]

The protected nonapeptide resin Boc-Tyr(BrZ)-Pro-Phe-Gly-BHA was prepared according to Example 13 on a 1 mmol scale. After removal of the N-terminal Boc group with 50% TFA in methylene chloride and neutralizing the resulting TFA salt with 5% DIEA in methylene chloride, the peptide was cleaved from the resin with removal of the side chain protecting groups by treatment with anhydrous liquid HF (10 mL) in the presence of anisole (1 mL) at 0°C for 1 hour. After removal of the HF under vacuum, the resin was washed with ethyl ether and air-dried. The resin was then extracted with glacial acetic acid and the combined extracts were lyophilized to yield a crude peptide.

The resulting crude peptide was purified by a bond elute column (A: 0.1% aqueous TFA B: CH_3CN-0.1% TFA, 0-30% B) to give 80 mg of the title compound. MS (ES) m/e 482 $[M+H]^+$; HPLC k' 4.47 (PRP-1, gradient, A: 0.1% aqueous TFA B: CH_3CN-0.1% TFA, 5-50% B over 30 minutes, UV detection at 220 nm).

Example 4

Preparation of H-Tyr-Pro-Trp-Gly-NH_2 [SEQ ID NO:2]

The protected nonapeptide resin Boc-Tyr(BrZ)-Pro-Trp-Gly-BHA was prepared according to Example 13 on a 1 mmol scale. After removal of the N-terminal Boc group with 50% TFA in methylene chloride and neutralizing the resulting TFA salt with 5% DIEA in methylene chloride, the peptide was cleaved from the resin with removal of the

side chain protecting groups by treatment with anhydrous liquid HF (10 mL) in the presence of anisole (1 mL) at 0°C for 1 hour. After removal of the HF under vacuum, the resin was washed with ethyl ether and air-dried. The resin was then extracted with glacial acetic acid and the combined extracts were lyophilized to yield a crude peptide.

The resulting crude peptide was purified by prep HPLC (PRP-1, gradient, A: 0.1% aqueous TFA B: CH_3CN-0.1% TFA, 5-50% B over 20 minutes) to give 75 mg of the title compound. MS (ES) m/e 521 $[M+H]^+$; HPLC k'4.69 (PRP-1, gradient, A: 0.1% aqueous TFA B: CH_3CN-0.1% TFA, 5-50% B over 30 minutes, UV detection at 220 nm).

Example 5

Preparation of H-Tyr-(N-Me)Ala-Leu-Gly-NH_2 [SEQ ID NO:15]

A. H-(N-Me)Ala-Leu-Gly-NH_2

A solution of H-Leu-Gly-NH_2 (536 mg, 2.00 mmol; Bachem Bioscience Inc.) in DMF was treated with Boc-(N-Me)-Ala-OH (446 mg, 2.20 mmol), HOBt (297 mg, 2.20 mmol), DIEA (1.40 mL, 8.00 mmol) and EDC•HCl (420 mg, 2.20 mmol) at room temperature for 24 hours. The reaction mixture was evaporated and the residue was taken up in EtOAc and extracted with 100 mL of 5% citric acid followed by 5% $NaHCO_3$. The material was then treated with 4N HCl/dioxane at room temperature for 30 minutes. The reaction was evaporated and evaporated from toluene to give 660 mg (2.16 mmol) of the above compound. MS(ES) m/e 273 $[M+H]^+$, 271 $[M-H]^-$.

B. Boc-Tyr-(N-Me)Ala-Leu-Gly-NH_2

The compound of Example 5A (660 mg, 2.16 mmol) in DMF was treated with Boc-Tyr-OH (666 mg, 2.37 mmol), HOBt (320 mg, 2.37 mmol), DIEA (1.54 mL, 8.64 mmol) and EDC•HCl (453 mg, 2.37 mmol) at room temperature for 24 hours. The reaction mixture was evaporated and the residue was taken up into EtOAc and extracted with 100 mL of 5% citric acid followed by sat. NaCl. The material was then purified by flash chromatography (silica gel, 9:1 chloroform:methanol) to give 140 mg (0.27 mmol) of the titled compound. MS(ES) m/e 536 $[M+H]^+$, 534 $[M-H]^-$.

C. H-Tyr-(N-Me)Ala-Leu-Gly-NH_2

The compound of Example 5B (140 mg) was treated with 4N HCl/dioxane at room temperature for 1 hour. The reaction mixture was evaporated and evaporated from toluene. Purification by prep HPLC (PRP-1, 5-30% gradient over 20 minutes of CH_3Cn in H_2O with 0.1% TFA) gave 100 mg of the title compound. MS (ES) m/e 436 $[M+H]^+$; HPLC k' 3.43 (PRP-1, gradient, A: 0.1% aqueous TFA B: CH_3CN-0.1% TFA, 5-50% B over 30 minutes, UV detection at 220 nm).

Example 6

Preparation of H-Tyr-Ala-Leu-Gly-NH_2 [SEQ ID NO:10]

A. H-Ala-Leu-Gly-OMe

A solution of H-Leu-Gly-OMe (330 mg, 1.63 mmol) in DMF was treated with Boc-Ala-OH (340 mg, 1.80 mmol), HOBt (243 mg, 2.00 mmol), DIEA (627 microliters, 3.60 mmol) and EDC•HCl (343 mg, 2.00 mmol) at room temperature for 24 hours. The reaction mixture was evaporated and the residue was taken up into EtOAc and extracted with 100 mL of 5% citric acid followed by 5% $NaHCO_3$. The material was then treated with 4N HCl/dioxane at room temperature for 30 minutes. The reaction was evaporated and evaporated from toluene to give 500 mg (1.61 mmol) of the title compound.

B. Boc-Tyr-Ala-Leu-Gly-OMe

The compound of Example 6A (500 mg, 1.61 mmol) in DMF was treated with Boc-Tyr-OH (500 mg, 1.77 mmol), HOBt (240 mg, 1.77 mmol), DIEA (620 microliters, 3.50 mmol) and EDC•HCl (339 mg, 1.77 mmol) at room temperature for 24 hours. The reaction mixture was evaporated and the residue was taken up into EtOAc and extracted with 100 mL of 5% citric acid followed by 5% $NaHCO_3$. The material was then purified by flash chromatography (silica gel, 100% EtOAc) to give 430 mg (0.802 mmol) of the titled compound. MS(ES) m/e 537 $[M+H]^+$, 535 $[M-H]^-$.

C. H-Tyr-Ala-Leu-Gly-NH_2

The compound of Example 6B (430 mg) was treated with 10 mL of 2M NH_4/MeOH at room temperature for 48 hours. The reaction was evaporated and treated with 4N HCl/dioxane at room temperature for 1 hour. The reaction mixture was evaporated

and evaporated from toluene. Purification of prep HPLC (PRP-1, 5-50% gradient over 20 minutes of CH_3CN in H_2O with 0.1% TFA) gave 126 mg of the title compound. MS (ES) m/e 422 $[M+H]^+$, 420 $[M-H]^-$; HPLC k' 2.98 (PRP-1, gradient, A: 0.1% aqueous TFA B: CH_3CN-0.1% TFA, 5-50% B over 30 minutes, UV detection at 220 nm).

Example 7

Preparation of H-(p-Amino-Phe)-Pro-Leu-Gly-NH_2 [SEQ ID NO:7]

A. Boc-Phe(4-Cbz-NH)-Pro-Leu-Gly-NH_2

A solution of H-Pro-Leu-Gly-NH_2 (100 mg, 0.341 mmol); Bachem Bioscience Inc.) in DMF was treated with Boc-Phe(4-Cbz-NH)-OH (100 mg, 0.375 mmol), HOBt (51 mg, 0.375 mmol), DIEA (119 microliters, 0.682 mmol) and EDC•HCl (72 mg, 0.375 mmol) at room temperature for 48 hours. The reaction mixture was evaporated and the residue purified by flash chromatography (silica gel, 5% MeOH in $CHCl_3$) to give 266 mg (0.407 mmol) of the title compound. MS(ES) m/e 681 $[M+H]^+$, 679 $[M-H]^-$.

B. H-(p-Z-Amino-Phe)-Pro-Leu-Gly-NH_2

The compound of Example 7A (266 mg) was treated with 4N HCl/dioxane at room temperature for 40 minutes. The reaction mixture was evaporated and evaporated from toluene. The mixture was treated with H_2 and 5% Pd/C under Parr conditions for 3 hours. The reaction was filtered through celite and purified by a bond elute column (0.1% aqueous TFA) to give 80 mg of the title compound. MS(ES) m/e 447 $[M+H]^+$, 445 $[M-H]^-$: HPLC k' 1.87 (PRP-1, gradient, A:0.1% aqueous TFA B: CH_3CN-0.1% TFA, 5-50% B over 30 minutes, UV detection at 220 nm).

Example 8

Preparation of H-(4-Azido-Phe)-Pro-Leu-Gly-NH_2 [SEQ ID NO:9]

The compound from Example 7B (16 mg) was treated with 3 mL 0.1N HCl and 54 microliters (0.54 mmol) $NaNO_2$ at 0°C for 10 minutes after which time 360 mL (0.360 mmol) NaN_3 was added. The reaction mixture reacted at 0°C for 1 hour and evaporated. The reaction was purified by prep HPLC (PRP-1, 10-30% gradient over 20 minutes of CH_3CN in H_2O with 0.1% TFA) to give 1.9 mg of the title compound. MS(ES) m/e 473 $[M+H]^+$, 471 $[M-H]^-$; HPLC k' 5.02 (PRP-1, gradient. A: 0.1% aqueous TFA B: CH_3CN-0.1% TFA, 5-50% B over 30 minutes, UV detection at 220 nm).

Example 9

Preparation of H-Tyr-hPro-Leu-Gly-NH_2 [SEQ ID NO:23]

A. Boc-Leu-Gly-OCH_3

A mixture of Boc-Leu-OH•H_2O (12.47 g, 50.0 mmol, Advanced Chemtech), glycine methyl ester hydrochloride (6.28 g, 50.0 mmol, Schweizerhall), EDC•HCl (9.60 g, 50.0 mmol), HOBt (6.78 g, 50.0 mmol) and DIEA (35.2 mL, 200 mmol) was stirred in 300 mL of DMF under argon for 18 hours. The solvent was then removed at reduced pressure. The residue was treated with 250 mL of water. The precipitate was then filtered and washed with water. The white resulting solid was recrystallized from chloroform/hexane to give 12.34 g (82%) of the title compound.

B. Boc-hPro-Leu-Gly-OMe

A solution of Boc-Leu-Gly-OMe of Example 9A (0.50 g, 1.6 mmol) was treated with 4 N HCl/dioxane at room temperature for 30 minutes. The reaction was evaporated. The residue was dissolved in DMF and treated with Boc-hPro-OH (370 mg, 1.6 mmol), HOBt (220 mg, 1.6 mmol), EDC•HCl (310 mg, 1.6 mmol) and DIEA (1.11 mL, 6.4 mmol) at room temperature for 24 hours. The reaction was evaporated, washed with 50 mL of H_2O and crystallized from chloroform/diethyl ether (2 mL/20 mL) to give 0.51 g (71%) of the title compound.

C. Boc-Tyr-hPro-Leu-Gly-OMe

The compound of Example 9B (0.15 g, 0.36 mmol) was treated with 4N HCl/dioxane for 0.5 hours. After the reaction was evaporated to dryness, the residue in DMF was treated with Boc-Tyr-OH (110 mg, 0.36 mmol), HOBt (50 mg, 0.36 mmol), EDC•HCl (70 mg, 0.36 mmol) and DIEA (0.25 mL, 1.44 mmol) at room temperature for 24 hours. The reaction mixture was evaporated, and the residue was washed with 20 mL of H_2O and recrystallized from chloroform/diethyl ether (1 mL/10 mL) to give 0.19 g of the titled compound.

D. H-Tyr-hPro-Leu-Gly-NH_2

The compound of Example 9C (0.18 g, 0.32 mmol) was treated with NH_3/MeOH solution (2.0 mmol, 20 mL) for 18 hours. The reaction was evaporated, and the residue was treated with HCl/dioxane (4N, 4 mL) for 1 hour. After the reaction was evaporated to dryness, the residue was further purified with HPLC (PRP column, 20% of CH_3CN in H_2O with 0.1% TFA) to give 86 mg of the titled compound. MS(ES) m/e $[M+H]^+$ 462; HPLC k' 5.01 (PRP-1, gradient, A: 0.1% aqueous TFA B: CH_3CN-0.1% TFA, 10-50% B over 30 minutes, UV detection at 220 nm).

Example 10

Preparation of H-Tyr-$^{\Delta 3,4}$Pro-Leu-Gly-NH_2 [SEQ ID NO:8]

A. Boc-$^{\Delta 3,4}$Pro-Leu-Gly-OMe

A solution of Boc-Leu-Gly-OMe from Example 9A (0.50 g, 1.6 mmol) was treated with 4N HCl/dioxane at room temperature for 30 minutes. The reaction was evaporated. The residue was dissolved in DMF and treated with Boc-$^{\Delta 3,4}$Pro-OH (370 mg, 1.6 mmol), HOBt (220 mg, 1.6 mmol), EDC•HCl (310 mg, 1.6 mmol) and DIEA (1.11 mL, 6.4 mmol) at room temperature for 24 hours. The reaction was evaporated, washed with 50 mL of H_2O and crystallized from chloroform/diethyl ether (2 mL/20 mL) to give 0.51 g (71%) of the title compound.

B. Boc-Tyr-$^{\Delta 3,4}$Pro-Leu-Gly-OMe

The compound of 10A (0.25 g, 0.70 mmol) was treated with 4N HCl/dioxane for 0.5 hours. After the reaction was evaporated to dryness, the residue in

DMF was treated with Boc-Tyr-OH (210 mg, 0.70 mmol), HOBt (100 mg, 0.70 mmol), EDC•HCl (140 mg, 0.70 mmol) and DIEA (0.96 mL, 2.80 mmol) at room temperature for 24 hours. The reaction mixture was evaporated, and the residue was washed with 20 mL of H_2O and recrystallized from chloroform/diethyl ether (1 mL/10 mL) to give 0.31 g of the title compound.

C. **H-Tyr-$^{\Delta 3,4}$Pro-Leu-Gly-NH_2**

The above compound 10B (120 mg, 0.21 mmol) was treated with NH_3/MeOH solution (2.0 mmol, 20 mL) for 18 hours. The reaction was evaporated, and the residue was treated with HCl/dioxane (4N, 4 mL) for 1 hour. After the reaction was evaporated to dryness, the residue was further purified with HPLC (PRP column, 20% of CH_3CN in H_2O with 0.1% TFA) to give 57 mg of the titled compound. MS(ES) m/e $[M+H]^+$ 462; HPLC k' 4.88 (PRP-1, gradient, A: 0.1% aqueous TFA B: CH_3CN-0.1% TFA, 10-50% B over 30 minutes, UV detection at 220 nm).

Example 11

Preparation of H-Ala(2-Naphthyl)-Pro-Leu-Gly-NH_2 [SEQ ID NO:13]

A. **Boc-Ala(2-Naphthyl)-Pro-Leu-Gly-NH_2**

Boc-ala(2-Naphthyl)-OH (169 mg, 0.5 mmol) in DMF was treated with H-Pro-Leu-Gly-NH_2 (147 mg, 0.5 mmol), HOBt (77 mg, 0.5 mmol), EDC•HCl (96 mg, 0.5 mmol) and DIEA (0.43 mL, 2.0 mmol) at room temperature for 24 hours. The reaction mixture was evaporated. The residue was washed with H_2O and recrystallized from chloroform/diethyl ether (1 mL/10 mL) to give 0.17 g of the title compound.

B. **H-Ala(2-Naphthyl)-Pro-Leu-Gly-NH_2**

The compound of Example 11A (160 mg, 0.27 mmol) was treated with HCl/dioxane (4N, 4 mL) for 1 hour. After the reaction was evaporated to dryness, the residue was recrystallized twice from methanol/diethyl ether (1 mL/10 mL) to give 101 mg of the title compound. MS (ES) m/e $[M+H]^+$ 482; HPLC k' 4.77 (PRP-1, gradient, A: 0.1% aqueous TFA B: CH_3CN-0.1% TFA, 10-50% B over 30 minutes, UV detection at 220 nm).

Example 12

Preparation of H-Trp-Pro-Leu-Gly-NH_2 [SEQ ID NO:14]

A. Boc-Trp-Pro-Leu-Gly-NH_2

Boc-Trp-OH (152 mg, 0.5 mmol) in DMF was treated with H-Pro-Leu-Gly-NH_2 (Bachem, 147 mg, 0.5 mmol), HOBt (77 mg, 0.5 mmol), EDC•HCl (96 mg, 0.5 mmol) and DIEA (0.43 mL, 2.0 mmol) at room temperature for 24 hours. The reaction mixture was evaporated. The residue was washed with H_2O and recrystallized from chloroform/diethyl ether (1 mL/10 mL) to give 0.18 g of the title compound.

B. H-Trp-Pro-Leu-Gly-NH_2

The above compound 12A (170 mg, 0.29 mmol) was treated with HCl/dioxane (4N, 4 mL) for 1 hour. After the reaction was evaporated to dryness, the residue was recrystallized twice from methanol/diethyl ether (1 mL/10 mL) to give 113 mg of the titled compound. MS (ES) m/e $[M+H]^+$ 471; HPLC k' 4.47 (PRP-1, gradient, A: 0.1% aqueous TFA B: CH_3CN-0.1% TFA, 10-50% B over 30 minutes, UV detection at 220 nm).

Example 13

General Procedure for Solid Phase Peptide Synthesis

Other peptides of formula (I) may be prepared by the following method.

Peptide amides are synthesized by solid phase peptide synthesis using benzhydrylamine resin as the support. Protected amino acids are added sequentially starting from the carboxyl terminus until the desired sequence is obtained. The Boc group is used for protection of the alpha-amino group. Side chain functional groups are protected as follows: arginine and histidine, tosyl (Tos); cysteine, p-methylbenzyl (MeBn); serine and threonine, benzyl ether (Bn); lysine, p-chlorocarbobenzyloxy (ClZ); glutamic acid and aspartic acid, benzyl ester (OBn); tyrosine, p-bromocarbobenzyloxy (BrZ). Removal of the Boc group was accomplished by treatment with 50% TFA in methylene chloride. Neutralization of the amine-TFA salt was accomplished by treatment with 7% DIEA in methylene chloride. Amino acids were coupled to the growing peptide using 3 equivalents of Boc-amino acid

and 3 equivalents of HOBt) in DMF and 3 equivalents of dicyclohexylcarbodiimide (DCC) in methylene chloride. Completeness of coupling was checked by ninhydrin test and couplings were repeated as necessary. The general protocol is given below.

1.	Wash with methylene chloride	1 x 1 min.
2.	Wash with 50% TFA	1 x 1 min.
3.	Deblock with 50% TFA	1 x 20 min.
4.	Wash with methylene chloride	6 x 1 min.
5.	Neutralize with 7% DIEA	3 x 2 min.
6.	Wash with methylene chloride	4 x 1 min.
7.	Wash with dimethylformamide	2 x 1 min.
8.	Boc-AA + HOBt in DMF	do not drain
9.	DCC in methylene chloride	2 hours
10.	Wash with dimethylformamide	2 x 1 min.
11.	Wash with methylene chloride	3 x 1 min.

For attachment of the first (C-terminal) residue to the BHA resin, the synthesis was begun at step 5. For all subsequent amino acids, the synthesis was begun at step 1.

Example 14

Effect of glycinamide on a human lung carcinoma cell line.

In 12 well plates , A549 cells (ATCC CCL-185) were added at 125 cells/well in McCOY's medium with 20% fetal bovine serum in 0.3% agar. After 10 ten days at 37°C , 6% CO_2 , colonies of greater than 50 cells were counted.

A549 inhibition

glycinamide conc. (ug/mL)	CFU
0.01% BSA	16
10	17
20	18

Example 15

Effect of glycinamide on normal mouse bone marrow.

In 12 well plates , bone marrow from female BDF1 mice were added to McCOY's medium with 15% fetal bovine serum, 200U M-CSF (Cellular Products Inc.) in 0.3% agar. After 7 days at 37°C . 6% CO_2 colonies of 50 or more cells

Normal bone marrow inhibition

glycinamide conc. (ug/mL)	CFU-GM
0.01% BSA	25
10	2
20	1

Example 16

Glycinamide inhibition detected by micro-screen assay.

Dilutions of glycinamide are made in phosphate buffered saline (PBS) with 0.01% bovine serum albumin. Different concentrations of glycinamide were added to wells in a maximum volume of 25 microliters. Non adherent bone marrow cells (4 x 10^4 cells/ 200 ul) were added in agar with 250 Units of M-CSF. These plates were grown in 37°C incubators in a humidified atmosphere of 7% CO_2 for 5 days. All Micro-Inhibition plates were incubated with MTT for several hours and solubilized with SDS overnight. Plates were read at 570 nm with a reference of 750 nm. All values compared to wells containing media / agar/ and cells with no M-CSF growth factor.

micro-inhibition assay

glycinamide (ug/ml)	optical density (570nm – 750nm)
0.01% BSA	.312
44	-.007
9	-.002
2	-.011
.4	.05
.07	.183
.01	.267
.003	.243

Example 17

Effect of glycinamide on the expansion of LPP from Sca-1+ cells.

Sca-1+ cells ,purified from bone marrow from BDF1 female mice were cultured with IL-1, IL-3, IL-6, SCF, GM-CSF, G-CSF and varying concentrations of glycinamide. After incubation at 37°C in 5% O_2 and 6% CO_2 , cultures were assayed for LPP at 4,7,10 and 13 days after start of incubation.

LPP expansion

days after start of culture	ug/mL glycinamide				
	0	7.5	15	30	60
4	6667	5169	2698	2627	1947
7	6947	17631	26573	8524	11000
10	16591	25924	25836	19258	13778
13	6111	33778	46036	71111	56778

Example 18

Effect of glycinamide on the expansion of HPP from Sca-1+ cells.

Sca-1+ cells ,purified from bone marrow from BDF1 female mice were cultured with IL-1, IL-3, IL-6, SCF, GM-CSF, G-CSF and varying concentrations of glycinamide. After incubation at 37°C in 5% O_2 and 6% CO_2 , cultures were assayed for HPP at 4,7,10 and 13 days after start of incubation.

HPP expansion

days after start of culture	ug/mL glycinamide				
	0	7.5	15	30	60
4	249	169	351	164	476
7	0	0	3889	3716	2444
10	1036	2076	0	0	2298
13	0	0	0	0	0

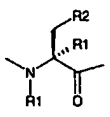
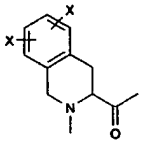
CLAIMS

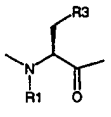
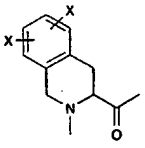
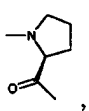
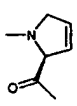
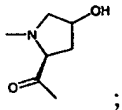
1. A method for expanding the population of myelopoietic progenitor cells *in vitro* comprising adding to a culture medium containing hematopoietic growth factors an amount of glycinamide or a peptide of formula (I):

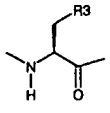
H-A-B-C-Gly-NH_2

(I)

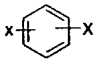
wherein:

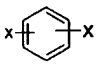
A is a bond,  , or  ;

B is a bond,  ,  ,  ,  , or  ;

C is a bond or  ;

R1 is H or C_{1-4}alkyl;

R2 is H,  , naphthyl, indoyl, or pyridyl;

R3 is H, C_{1-4}alkyl, cycloC_{3-8}alkyl,  , naphthyl, indoyl, or pyridyl; wherein the C_{1-4} alkyl may be substituted by one or more X groups;

X is H, OH, I, Br, NH_2, N_3, O-C_{1-4}alkyl, C_{1-4}alkyl, or COPh;

or a pharmaceutically acceptable salt thereof.

2. A method according to Claim 1 wherein the compound of Formula (I) is removed after 1-100 days and the cells are then transplanted into a subject in need thereof..

3. A method according to claim 1 wherein the growth factor is selected from the group consisting of M-CSF, IL-1, IL-3, IL-6, IL-11, G-CSF, GM-CSF, LIF, TGF-β, MIP1-α, FLT3 ligand, SCF, TPO, MPIF-1, IL-3/GM, IL-3/G, FLT3/GM or a combination thereof.

4. A method according to Claim 3 wherein the growth factor is IL-1, IL-3, SCF, or M-CSF.

5. A method according to Claim 3 wherein the amount of glycinamide is about 7.5 micrograms/mL to about 60 micrograms /mL.

6. The method according to claim 1 wherein said myelopoietic colonies are CFU-C colony forming cells.

7. The method according to claim 6 wherein said CFU-C colonies are CFU-GM colony forming cells.

8. A method for reversibly inhibiting myelopoiesis in mammalian tissue *in vivo* and *ex vivo* comprising administering to said tissue an amount of glycinamide or a peptide of formula (I) as defined in Claim 1.

9. The method according to claim 8 wherein said myelopoietic colonies are CFU-C colony forming cells.

10. The method according to claim 9 wherein said CFU-C colonies are CFU-GM colony forming cells.

11. The method according to claim 8 wherein said administering step comprises administering glycinamide to a mammalian subject and wherein said reversible inhibition occurs *in vivo*.

12. The method according to claim 8 wherein said administering step comprises administering a compound of Formula (I) to a mammalian tissue *ex vivo* and wherein said reversible inhibition occurs *ex vivo*.

13. The method according to claim 8 wherein said administering step comprises administering a compound of Formula (I) to a mammalian subject and wherein said reversible inhibition occurs *in vivo*.

14. The method according to claim 8 wherein said administering step comprises administering a compound of Formula (I) to a mammalian tissue *ex vivo* and wherein said reversible inhibition occurs *ex vivo*.

15. A method for identifying or screening for compounds which inhibit CFU-C formation of myeloid progenitor cells which comprises the steps of:

(a) bringing together a test sample containing one or more test compounds and hematopoietic growth factors with a preparation comprising myeloid progenitor cells in soft agar;

(b) incubating said test sample and said preparation under conditions which would permit the detection of inhibition of CFU-C colony growth; and

(c) determining the degree of inhibition caused by said test sample by measuring MTT conversion by extracting said preparation and measuring optical density at 570 nm with a reference filter of 750 nm on an ELISA reader.

16. The method according to claim 15 wherein said preparation comprises isolated myeloid progenitor cells.

17. The method according to claim 16 wherein said preparation is bone marrow cells.

18. The method according to claim 15 wherein said preparation is mobilized peripheral blood stem cells.

19. The method according to claim 1 wherein the compunds are chosen from the group consisting of:

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: SMITHKLINE BEECHAM CORPORATION

(ii) TITLE OF THE INVENTION: COMPOSITIONS AND METHODS FOR EXPANSION OF HEMATOPOIETIC CELLS

(iii) NUMBER OF SEQUENCES: 23

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: SmithKline Beecham Corporation
(B) STREET: 709 Swedeland Road
(C) CITY: King of Prussia
(D) STATE: Pennsylvania
(E) COUNTRY: USA
(F) ZIP: 19406

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/090,019
(B) FILING DATE: 19-JUN-1998

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Hall,Linda
(B) REGISTRATION NUMBER: 31,763
(C) REFERENCE/DOCKET NUMBER: P50791

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (610)270-5016
(B) TELEFAX: (610)270-5090
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

His Tyr Pro Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

His Tyr Pro Trp Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

His Tyr Pro Phe Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

His Phe Pro Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

His Pro Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

His Ile Tyr Pro Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

His Asn His Phe Pro Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

His Tyr Pro Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Asn Asn Phe Pro Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

His Thr Ala Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

His Thr Pro Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

His Asx Phe Pro Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

His Asn Pro Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

His Trp Pro Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

His Tyr Asn Met Ala Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

His Ile Tyr Pro Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

His Pro Pro Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

His Tyr His Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

His Met Tyr Pro Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

His Thr His Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

His Asx Pro Leu Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

His Tyr Pro Lys Gly Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

His Tyr Pro Leu Gly Asn His
1 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/14065

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01N 1/02; C12N 15/85; C07K 5/00; A61K 35/00
US CL : 435/1.1, 2, 325; 424/93.1; 530/300.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/1.1, 2, 325; 424/93.1; 530/300.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, CAPLUS, WPIDS, HCAOLD, HCAPLUS, REGISTRY

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	HOROWITZ et al. Ex vivo expansion of hematopoietic cells with glycinamide. Blood. 15 November 1998, Vol. 92, No. 10, Suppl. 1, Part 1-2, pp. 311B.	1-18
P, X	CALLAHAN et al. Identification of glycinamide as the active prinicipal responsible for the effects of Tyr-MIF on hematopoietci progenitor cells. Blood. 15 November 1998, Vol. 92, No. 10, Supppl 1, Part 1-2, pp. 581A.	1-18
A	BHATNAGAR et al. Structure-activity relationships of novel hematoregulatory peptides. Journal of Medicinal Chemistry. September 1996, Vol. 39, No. 19, pages 3814-3819.	1-18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 16 AUGUST 1999	Date of mailing of the international search report 21 OCT 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer CARRIE MARLENE STROUP Telephone No. (703) 306-5439

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/14065

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0637448 A1(GHIRARDI, ISCOFAR SAS DI PAOLO E.) 08 February 1995, Table 2, page 4.	1-19
A	SMITH et al. Inhibition of pluripotent embryonic stem cell differentiation by purified polypeptides. Nature. 15 December 1988. Vol. 336, No. 6200, pages 688-690, especially abstract.	1-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/14065

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 19
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

the last page is missing from the list of claims.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.